J. of Supercritical Fluids 56 (2011) 243–248

Contents lists available at ScienceDirect

The Journal of Supercritical Fluids

journal homepage: www.elsevier.com/locate/supflu

SFE from *Bidens pilosa* Linné to obtain extracts rich in cytotoxic polyacetylenes with antitumor activity

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ARTICLE INFO

Article history:
Received 2 June 2010
Received in revised form
18 December 2010
Accepted 19 December 2010

Keywords:
Bidens pilosa
Polyacetylenes
Antitumor
Supercritical fluid extraction

ABSTRACT

Bidens pilosa L. is a plant considered medicinal by some South American cultures. It contains polyacetylenes which may be the constituents responsible for its antitumor activity. Extracts obtained by hydroethanol maceration (HCE) and supercritical fluid extraction (SFE) were monitored for antitumor activity and the presence of polyacetylenes in the constitution. Both extracts killed concentration-dependently the MCF-7 cells in culture, although the SFE extract presented superior cytotoxic activity. The SFE presented IC_{50} = 437 (428–446) μg/mL in 24 h of incubation, decreasing to IC_{50} = 291 (282–299) μg/mL at 48 h. The HCE started causing DNA cleavage at 160 μg/mL while the SFE extract started at 40 μg/mL, a concentration enough to initiate the *in vitro* cleavage. The presence of polyacetylenes as the major compounds in SFE was confirmed by TL chromatography combined with UV–vis analyses. Ehrlich ascites carcinoma-bearing mice were used for the antitumor study. Animals were divided in five groups: normal, negative control, positive control (Doxorubicin 0.06 mg/kg), test group HCE and test group SFE (100 mg/kg b.w. per day). After 9 days of treatment, 50% of randomly chosen animals from each group were sacrificed for the study. The parameters evaluated were: body weight, abdominal circumference, volume of ascitic fluid and tumor cells, viable and nonviable tumor cell count, determination of mean survival time and increased life-span. Both extracts presented antitumor activity, but SFE reduced more the volumes of ascites fluid and the tumor cells (4 ± 1 and 1 ± 0.4 mL, respectively), while caused higher mean survival time (17 days) and increased life span (~31%). The results suggest the importance of the polyacetylenes from *B. pilosa* as leader molecules to contribute to a new anticancer drug by using the supercritical technology.

1. Introduction

Since the ancient times, the plants have been used for medicinal purposes. The plant kingdom is indeed responsible for the largest share of chemical diversity known and recorded in the literature [1]. Actually, chemical and pharmacological studies involving isolated compounds and other plant derived ones, they have appeared in the scientific set for therapeutic activities despite the high costs of the researches and the synthetic drug development [2]. *Bidens pilosa* Linné (Asteraceae) is a medicinal plant with a wide occurrence in the tropical regions. It has a long history of popular use by Amazonian Indians, Caribbean, Cuban folks and Chinese medicine for several reasons including the treatment of tumors, malaria and liver diseases. The hydroethanol crude extract and particularly its chloroform fraction already demonstrated to inhibit the Ehrlich ascites carcinoma *in vitro* and in mice [3–5]. Previous studies have revealed that the plant presents a broad phytochemical constitution with little peculiarities according to the place where it is harvested. The major compounds already identified are flavonoids and polyacetylenes [6].

Taking *B. pilosa* as a potential source for a novel useful drug to treat cancer, a special interest arose for its polyacetylenic compounds. The polyacetylenes comprise a relative rare class of long chain acetylene-derived compounds. They are often unstable hydrocarbons that strongly absorb long-wave UV radiation; their activities may be altered upon exposure to light (photoactivation). Considering acetylenes are often very reactive gases, it is interesting to realize how polyacetylenes may be sufficiently stable to be extracted, isolated and characterized through phytochemical standard techniques. Actually, nowadays more than 1000 polyacetylenes are now known as plant products [7,8]. They contain unique carbon–carbon triple bond functionality, which is intriguing for their wide variety of activities and economic potential. Most polyacetylenes are poisonous and they have been considered phy-

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doi:10.1016/j.supflu.2010.12.011

toalexins. Worldwide some groups of researchers have identified and isolated different polyacetylenes from *B. pilosa* cultivated in different parts of the globe [5,9–11]. Cytotoxicity effects on parasites [5] and modified cells in culture [10] have ever been reported for some polyacetylenes.

Based on the mentioned above, one might expect the variety of polyacetylenes in the plant could be the main responsible for the antitumor activity. Supercritical fluid extraction was already employed with success to extract polyacetylenes from *Atractylodes japonica* [12]. Accordingly, in this work hydroethanol maceration and supercritical fluid extraction were performed and monitored by phytochemical techniques and biological assays for antitumoral activity. The results were then compared in order to evaluate the efficacy of the process to extract polyacetylenes from samples of *B. pilosa* while preserving their functionality.

2. Experimental procedures

2.1. Plant material

B. pilosa Linné (Asteraceae) was collected at the Jureia Botanical Reserve (Atlantic Forest), São Paulo State, Southeast Brazil. The plant was identified by Inês Cordeiro, a voucher specimen (M.H. Rossi SP384167) was deposited at the Herbarium do Estado Eneida P.K. Fidalgo of the Botanical Institute, São Paulo, Brazil. This study followed the international, Brazilian and institutional rules concerning the biodiversity rights.

2.2. Extraction

The dried aerial parts of the plant were powdered and exhaustively extracted with ethanol–water solution (9:1) at room temperature for 3 days (3 times). The solvent was eliminated under low pressure to obtain a dried hydroethanol crude extract (HCE) yielding 8.3% (w/w) in terms of starting material. The supercritical extract (SFE) was prepared using a dynamic extractor unit. A mass of 15 g of dried and milled plant aerial parts was placed inside the extractor to form a bed of particles under controlled conditions of temperature and pressure [13,14]. The supercritical fluid extraction was performed with carbon dioxide (CO_2) as solvent and maintaining the extraction conditions at 40 °C, 250 bar and solvent flow rate of 15.0 ± 0.5 g/min for 240 min. The process used CO_2 99.9% pure (White Martins, Brazil) delivered at pressure up to 60 bar. The obtained extract was collected in amber flasks and weighed in an analytical balance (OHAUS, Model AS200S, NJ, USA). The procedure yielded 2% in terms of starting material and it was repeated until obtaining material enough for the biological assays.

2.3. Phytochemical analyses

Dried SFE was solubilized in ethyl acetate PA to be chromatographed by semi preparative thin layer chromatography carried out on silica gel 60 F_{254} (Merck, Darmstadt) using EtOAc–H_2CO_2–AcOH–H_2O (750:5:5:20) as mobile phase. The material of the major spot was collected and resolubilized in methanol PA. After centrifugation, the supernatant solution was submitted to UV–vis spectrophotometrical analyses. Data of spectra were then compared to literature [5,7].

2.4. Cytotoxicity in vitro

Preliminarily HCE and SFE were assessed for cytotoxicity effects *in vitro* on breast cancer cell line (MCF-7). The cells were grown in DMEM medium supplemented with 10% fetal calf serum, penicillin (100 U/mL) streptomycin (100 μg/mL) and gentamycin (50 μg/mL) in a humidified incubator with a 5% CO_2 atmosphere at 37 °C. The cells were plated onto 96-well plates at a density of 10^4 cells/well. Cells were allowed to set for 24 h and then the medium was replaced by other (200 μL) containing HCE or SFE (31.25–1000 μg/mL) initially solubilized in Tween 80 and then in DMEM. At first, the cells were exposed for 24 h. At the end of incubation an endpoint of cytotoxicity, the tetrazolium salt assay (MTT) was performed according to Mosmann [15]. The results were presented in the form of IC_{50} and/or percentage of cell viability. According to the previous results and in order to evaluate if the cytotoxicity was time-dependent, later the cells were exposed to the most cytotoxic extract when they were allowed to keep until 48 h under incubation.

2.5. Effects on the DNA

The effects to uncouple the integrity of DNA *in vitro* were assessed via standard electrophoresis in 0.8% agarose gel done with purified pUC9.1 plasmid DNA extracted from *Escherichia coli* DH5SαFIQ [16]. The substances able to cause DNA cleavage can generate breaks in the strands which change the conformation and the migration pattern in agarose electrophoresis. The intact DNA is found to be present at a supercoiled conformation (FI). Damage can cause a simple break in the double helix structure giving rise at first to an annealed structure of DNA strands (FII). A stronger damage is expected to be able to cause double breaks generating the linear conformation of strands (FIII). The kit Perfectprep Plasmidi Mini (Eppendorf®) was used to extract the plasmid DNA. For the evaluation 5 μL of a solution done with 600 ng of DNA were incubated at pH 7.4, 50 °C for 16 h with 45 μL of a solution done with HCE or SFE at 10–160 μg/mL in 25 mM PIPES buffer (piperazine-N,N0-bis[ethanesulfonic acid)] and 8.0 in 25 mM HEPES buffer [N-(2-hydroxyethyl)piperazine-N0-(2-ethanesulfonic-acid)]. Only vehicle was used for the negative control. Fe-EDTA (20 μM), an oxidative damage inducer solution was used for the positive control. All incubations were done in triplicates. After incubation, the DNA was loaded onto the gel at approximately 10 μL/well and subjected to electrophoresis at 70–80 V in TBE buffer (89 mM Tris borate, 2 mM EDTA) for 30–45 min. The gels were stained with ethidium bromide (0.5 μg/mL, 20 min). The resulting gels were digitalized by a photodocumentation system (UVP, CA, USA) and then the DNA bands were quantified.

2.6. Animals

Male isogenic Balb/c mice weighing 20 ± 5 g were housed under controlled conditions (12 h light–dark cycle, 22 ± 2 °C, 60% air humidity) and had free access to standard laboratory food and water. All animals were allowed to acclimatize for at least 5 days prior to the first treatment. Animals fasted for 12 h before experiments, but water was allowed *ad libitum*. All animal procedures were conducted in accordance with legal requirements appropriate to the species (NIH publication #80-23, revised in 1978) and with the local ethics committee approval (PP00338/CEUA and 23080.018827/2009-79/UFSC).

2.7. Antitumor effects

The activities of HCE and SFE were evaluated against the Ehrlich ascites carcinoma (EAC). Animals were divided into 5 groups ($n = 12$). The normal group was not inoculated with tumor cells, while the other ones were injected with EAC cells (0.2 mL of 5×10^6 cells/mice) intraperitoneally. The inoculation day was taken as day 0 and the experimental treatments started 24 h later. From the 1st day, the vehicle (water–Tween 80, 99:1) 50 μL/mouse per day was administered intraperitoneally to the normal and negative control groups. HCE, SFE (100 mg/kg, treated groups) and Doxoru-

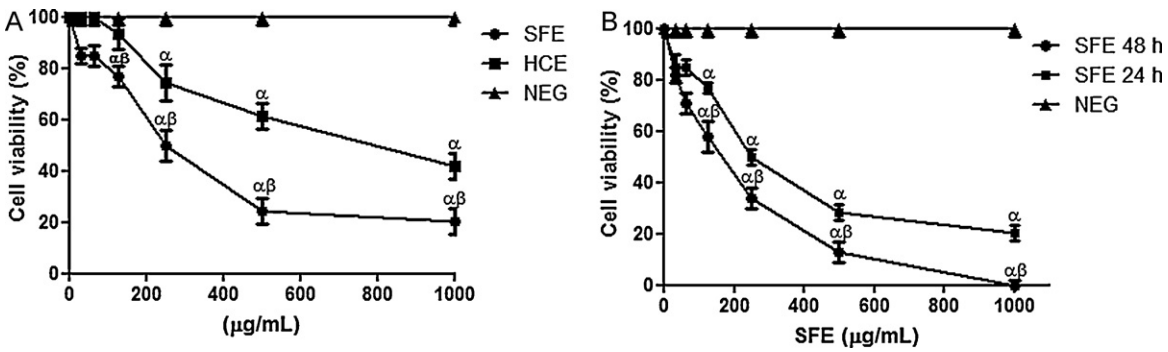

Fig. 1. Cytotoxicity of SFE and HCE on MCF-7 cells under 24 h of incubation (A); the cytotoxicity of SFE on MCF-7 cells after 48 h of incubation (B). (α) Denotes statistic differences compared to NEG and (β) compared to HCE in A and 24 h in B, respectively when $P > 0.05$.

bicin (0.06 mg/kg/day, positive control group), were administered during 9 days. 24 h after the last dose, 50% of randomly chosen animals from each group were sacrificed for the study of the antitumor activity. The remaining animals were kept to check the survival time [4]. Parameters of morphology and chronology change in the body weight and abdominal circumference, mean survival time (MST) and percentage of increased life span (% ILS) were measured. The MST and ILS were calculated using the following Eq. (1) and Eq. (2) according to Mazumder et al. [17]:

$$\text{MST} = \frac{\text{day of the first death} + \text{day of the last death}}{2} \quad (1)$$

$$\text{ILS}(\%) = \left[\left(\frac{\text{MST of treated group}}{\text{MST of control group}}\right) - 1\right] \times 100 \quad (2)$$

The effects of HCE and SFE were also assessed by the determination of the volume of ascites fluid, tumor cells volume and viable and nonviable tumor cell count by the Trypan blue incorporation method [18].

2.8. Statistical analyses

Most data were expressed as mean ± S.D. and/or confidence interval. Statistical analysis were performed to compare treated groups to respective control groups using evaluations by one-way analysis of variance (ANOVA) complemented with the Tukey–Kramer multiple comparison tests with equal sample size. Values of $P < 0.05$ were considered statistically significant.

3. Results and discussion

Some studies about *B. pilosa* have revealed that the plant presents a broad phytochemical constitution. Sometimes subtle diversities might be possibly observed according to the place where it is harvested, although some compounds are constantly present such as sesquiterpens, flavonoids, carbonates, hydrochlorides, proteins, esters and essential oils among phenyl and other polyacetylenes [10,11,19,20]. According to Geissberger and Sequin [11], the great assortment of constituents in *B. pilosa* may be the fact that rationalizes the broad spectrum of activities alleged by the popular medicine. Indeed, some protective effects, e.g. against liver injury, have already been reported to some constituents of *B. pilosa* like the flavonoid ones [3], while other constituents like the polyacetylenes for their nature are expected to posses more toxic activities [5,10]. As mentioned, there are evidences that demonstrate that the polyacetylenes may work as toxin and they must be the main responsible for the antitumor potential of the hydroethanol crude extract of *B. pilosa* and its chloroform fraction [4,7]. It is known that when an extraction process is initially performed with plant parts, according to the affinity with the solvents used, many kinds of substances, pharmacologically active or not, can be concomitantly extracted [21]. In this trial the supercritical fluid extraction method was performed and monitored for its efficacy to extract polyacetylenes from *B. pilosa* while preserving their functionality to combat the tumors. The supercritical technology has been receiving great attention for pharmaceutical applications due to the selectivity aspects of the process and due to the particular effect related to biological activity of the resulting extracts [22,23].

Initially, HCE and SFE were assayed for cytotoxicity on MCF-7 cells in cultures *in vitro*. The resulting data are shown in Fig. 1, which shows that at 24 h of incubation with the MCF-7 cells, both extracts (HCE and SFE) were able to kill the cells in a concentration-dependent manner when compared to the negative control. Data were collected by checking the levels of decreased cell viability through the MTT method and, then it was possible to calculate an IC_{50} value. It was found that, in 24 h of incubation, HCE presented IC_{50} = 811 (795–828) μg/mL while SFE was defined to be more cytotoxic. The levels of cell viability were found even lower when the cells were exposed to SFE under the same incubation time. Accordingly, the IC_{50} of SFE related to its activity in 24 h was defined to be 437 (429–446) μg/mL (Fig. 1(A)). In the following, to investigate whether the SFE cytotoxicity was additionally time-dependent, the MCF-7 cells were incubated with SFE at the same concentration range and then allowed to keep until 48 h when the cell viability was checked. Data shown in Fig. 1(B) demonstrated that the IC_{50} determined to SFE after 48 h was decreased significantly, with values of IC_{50} (48 h) = 291 (283–300) μg/mL. The behavior presented by the SFE extract, compared to the HCE extract, indicates the relevance of the selectivity of the supercritical process towards the components with cytotoxic activity, a performance equivalent to the antimicrobial activity was obtained by Michielin et al. for supercritical extracts of *Cordia verbenaceae* [22].

Once the cytotoxicity of the extracts HCE and SFE on MCF-7 cells was observed, the action mechanism that caused the cells death started to be considered. Among the many possible approaches, a study for direct actions on DNA was initially taken into account and therefore, in attempt to preliminarily achieve the mechanism of the cell damage, an evaluation of the effects *in vitro* on plasmid DNA was chosen to be performed. Some authors suggest that the substances able to cause DNA damage can make this because they are able to change the DNA conformation and its migration pattern. These substances have a nuclease activity and often they do can cause a stress on the DNA which promotes its cleavage that may occur through hydrolysis, oxidation, among other possibilities [24]. The DNA damage may trigger cell death [25].

Data in Fig. 2(A) show the plasmid DNA behavior under the effects of the controls treatment. The FI form is predominant in the intact DNA in the negative control (NEG). On the other hand, when

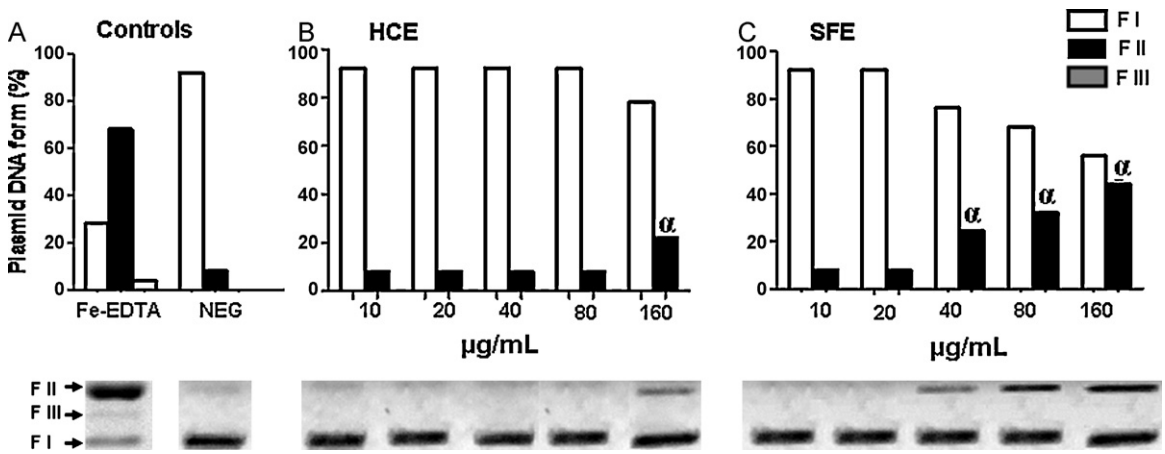

Fig. 2. Fe-EDTA DNA damage (A) and FI conformation in the intact DNA in the negative control (NEG) (A). HCE and SFE damage on the DNA (B and C). (α) Denotes statistic difference compared to NEG when $P > 0.05$.

the DNA was exposed to Fe-EDTA, it was possible to check deep alterations in the migration pattern, indicating Fe-EDTA was effective to cause damage. In the DNA exposed to Fe-EDTA it was found predominance of the FII form and additionally a presence of the FIII DNA form was detected. In Fig. 2(B and C), the data show how the DNA form was found after the exposition to HCE and SFE, respectively. It is possible to see that once again both extracts presented some toxicity. Data in Fig. 2(B) show that HCE was able to cause some DNA damage only at 160 μg/mL when compared to NEG, while data in Fig. 2(C) show that SFE at 40 μg/mL was enough to produce DNA damage. This performance demonstrates one more time the relevance of the extract obtained by supercritical extraction, compared to HCE, 1/4 of the amount of extract was able to produce an equivalent toxicity, indicating superior strength of the SFE, probably due to the higher concentration in desirable components. According to the obtained data presented in Fig. 2(C), although the FIII DNA form has not been reached, SFE presented significant effect stronger than HCE. SFE started to produce damage in DNA, changing the DNA migration pattern already at 40 μg/mL and thereafter, its effect started to increase dependent to its concentration when compared to NEG (Fig. 2(C)).

Toxicity *in vitro* and an antitumor potential in mice have already been reported for the hydroethanol crude extract from *B. pilosa* [4]. But the findings here started to indicate that these activities could be possibly improved by applying the supercritical fluid extraction procedure to obtain the active constituents. According to this observation, the SFE extract was considered even more active than HCE, and therefore this should mean that the active constituents were preserved during the supercritical process. Taking it into consideration, the SFE was then evaluated by thin layer chromatography combined with UV–vis spectrophotometric analyses to evaluate the efficacy of the supercritical fluid extraction to obtain and preserve polyacetylenes from samples of *B. pilosa.* Initially, the SFE extract was applied to semi preparative thin layer chromatography (TLC) performed in batch to evaluate some mobile phase systems. After defining the adequate mobile phase, three distinct main spots were checked. Sometimes, degradation dependent on the exposure to time and/or light was observed, indicating the relative instability of the substances present in the extract in analyses. That is why the UV–vis analyses were done immediately after the separation. A triplicate of the TLC running of SFE solubilized in ethyl acetate using EtOAc–H_2CO_2–AcOH–H_2O (750:5:5:20) as mobile phase is shown in Fig. 3(A). The material from the major spots, spot A was taken out of the layer and collected. The spot A substances were resolubilized in methanol PA and after centrifugation, the solution was submitted to spectrophotometrical UV–vis array analyses. The absorbance spectrum is shown in Fig. 3(B).

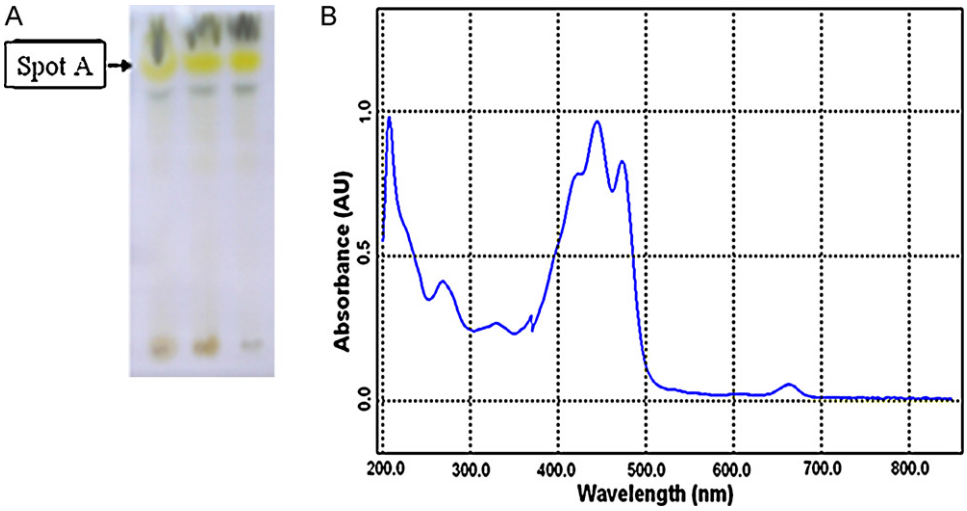

Fig. 3. Thin layer chromatography of SFE, the major spot A (A). The UV–vis spectra of substances from spot A in methanol PA with typical bands of polyacetylenes (B).

Table 1
Effects of *Bidens pilosa* treatments (HCE or SFE, 100 mg/kg) and Doxorubicin (0.06 mg/kg) in mice previously inoculated with Ehrlich ascites carcinoma and controls. Normal control group (NC), negative control group (NEG) and positive control group treated with Doxorubicin (DOX). *Note*: Data expressed as the mean ± S.D., $n = 6$ (α) means significant differences compared to NEG, (β) significant differences compared to HCE ($P < 0.05$).

Parameter/group	NC	NEG	HCE	SFE	DOX
Increase in body weight (g)	1 ± 1	8 ± 1	7 ± 2	$5 \pm 1^{\alpha}$	$3 \pm 1^{\alpha\beta}$
Increase in abdominal circumference (cm)	<1	4 ± 1	$1 \pm 1^{\alpha}$	$1 \pm 1^{\alpha}$	$<1^{\alpha\beta}$
Ascites fluid (mL)	–	10 ± 1	$7 \pm 1^{\alpha}$	$4 \pm 1^{\alpha\beta}$	$2 \pm 1^{\alpha\beta}$
Tumor cells (mL)	–	4 ± 1	$2 \pm 1^{\alpha}$	$1 \pm 1^{\alpha}$	$< 1^{\alpha\beta}$
Nonviable/viable tumor cell count	–	<1	<1	$1 \pm 1^{\alpha\beta}$	$2 \pm 1^{\alpha\beta}$
MST (days)	30	13	15^{α}	$17^{\alpha\beta}$	$20^{\alpha\beta}$
ILS (%)	100	0	15^{α}	$31^{\alpha\beta}$	$54^{\alpha\beta}$

Data were then compared to literature [5,7]. The UV–vis spectrophotometrycal analyses confirmed the presence of substances majoritarily with polyacetylenes absorbance behavior in the SFE sample. The maximum absorbance peaks were recorded at λ_{max} (nm): 208, 269, 330, 370, 445, 473 and 667. The supercritical fluid extraction appeared to be an alternative operation to produce an extract with improved anticancer activity as confirmed in the following data. Therefore, the results pointed to preservation of the triple bonds in the polyacetylenes by employing supercritical carbon dioxide as the extraction solvent. The obtained data are according with the antitumor activity of SFE *in vivo* presented in Table 1.

The antitumor effects of HCE and SFE were evaluated comparatively against the EAC in mice. EAC is a transplantable tumor. When it is inoculated in the peritoneum of animals it causes a regular rapid development of a tumor ascites. In EAC-bearing mice, the ascites fluid is the direct nutritional source for the maintenance of the tumor cells. It contains several growth factors and the nutrients needed for the cell survival and proliferation. It is the site where the EAC grow in suspension [26]. Therefore, in this case the measure of the tumor cell volume is that which correspond to the real tumor size. In general, one would expect that an antitumor activity should mean reduction in the both measures.

The data obtained after the 9-day treatment of EAC-bearing mice with HCE, SFE (100 mg/kg/day) or the positive control Doxorubicin (0.06 mg/kg/day) on body weight, mean survival time, volume of ascitic fluid and tumor cells, tumor cell count (viable and nonviable cells) are shown in Table 1. All the treatments reduced significantly the change in the body weight, the volume of ascitic fluid and tumor cells when compared to the negative control (NEG). Furthermore, the proportion of nonviable/viable tumor cells was found increased when the treatments were done with HCE, SFE or Doxorubicin when compared to NEG. According to the obtained data, the SFE extract promoted higher reduction in the volume of ascites fluid when compared to HCE. Besides, it caused higher inhibition of tumor growth in basis of increasing in abdominal circumference (Fig. 4). SFE's activity was closer to the Doxorubicin's activity.

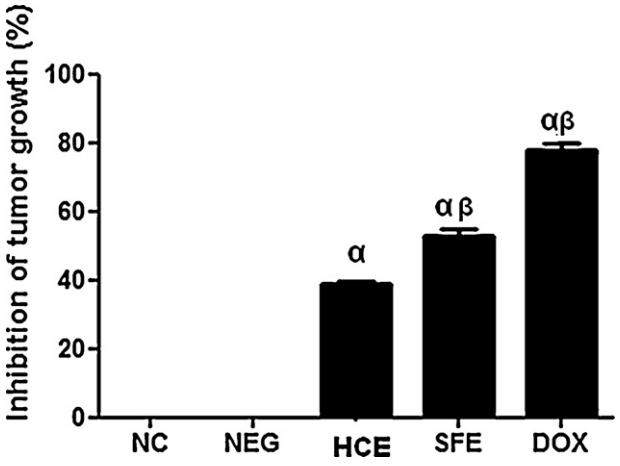

Fig. 4. Inhibition of tumor growth in basis of increasing in abdominal circumference. (α) Denotes statistic differences compared to NEG and (β) denotes statistic differences compared to HCE when $P > 0.05$.

According to Clarkson and Burchenal [27], one of the most reliable criterions for judging the value of any antitumor drug is the prolongation of the life span of animals. In this sense, it is worthwhile to remark HCE, SFE and Doxorubicin treatments increased both indexes the mean survival time and the percentage of increased life-span when compared to NEG as depicted in Table 1. It is very possible the recovery of normality of the morphological parameters caused by the treatments finally resulted in the prolongation of the survival time. Considering these parameters, it is possible to affirm again SFE was more active than HCE to combat the tumor. Animals treated by SFE lived about 31% more than animals from the negative control, while HCE produced about 15% of increase life-span (Table 1). SFE caused higher mean survival time and higher percentage of increased life span when compared to HCE while Doxorubicin showed again the highest indexes increase (MST = 20 days and ILS = 54%) (Table 1).

4. Conclusions

From the overall results, we can conclude that *B. pilosa* has antitumor constituents active against the MCF-7 cells and the Ehrlich ascites carcinoma. Among the main responsible constituents for the plant activity are the polyacetylenic compounds which may interact with the DNA to cause its cleavage. The polyacetylenes from the plant presented a nuclease activity *in vitro*. Considering that there was an improvement in the biological activity when the extract obtained by SFE was employed, compared to HCE, the use of high pressured carbon dioxide to extract antitumor constituents from *B. pilosa*, in this case, was confirmed to be very suitable. Consequently, the results obtained appear to support that the polyacetylenes from *B. pilosa* may present effects that justify their consideration to be evaluated like leader molecules or prototypes in the development of a useful new anticancer drug.

Acknowledgments

This research was supported by grants from CNPq/MCT-Brazil. RC Pedrosa (Proc. 309438/2008-0) and SR Ferreira (Proc. 474765/2007-5) are recipients of research grants from the Conselho Nacional de Pesquisa (CNPq), Brazil. MR Kviecinski, KB Felipe and P Benelli are fellows of CAPES/CNPq-Brazil.

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